8-2004

Atmospheric Plasma Inactivation of Foodborne Pathogens on Fresh Produce Surfaces

Faith Michelle Johnson
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Faith Michelle Johnson entitled "Atmospheric Plasma Inactivation of Foodborne Pathogens on Fresh Produce Surfaces." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in .

David A. Golden, Major Professor

We have read this thesis and recommend its acceptance:

P. Michael Davidson, John R. Mount

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

Anne Mayhew
Vice Chancellor for Academic
Affairs and Dean of Graduate Studies

(Original signatures are on file with official student records)

Atmospheric plasma inactivation of foodborne pathogens on fresh produce surfaces

A Thesis Presented
for the Master of Science Degree
The University of Tennessee, Knoxville

Faith Michelle Johnson
August, 2004

Acknowledgements

I would like to thank my major professor, David Golden, for teaching me something new every day and giving me the opportunity to complete my M.S. degree. He has always challenged me to go beyond what was expected and truly get the most out of my education. He has become both a mentor and a friend, and I really appreciate him taking me under his wing. I would also like to thank Kim Stanley and Nancy DeTrana for their encouragement, friendship, and support with everything over the past year. They have become a second family, and I cannot imagine how I would have made it without them.

I would like to express my appreciation to Atmospheric Glow Technologies, in specific, Dr. Kimberly Kelly-Wintenberg, Suzanne South, and Daniel Sherman. It was very exciting to do research with the innovative technology that has been developed at AGT.

The Food Science Department as a whole has my appreciation. All of the faculty, staff, and students are of the highest caliber and make this department one of the best on campus. Everyone makes this an enjoyable environment for both working and learning.

Last, but certainly not least, I would like to thank my fiancé, Rob Critzer, and my family for their endless support. Whenever it seemed as if the sky was falling they were always there to ensure that it was not and to help in any way they could. I really appreciate everything they have sacrificed to get me where I am today.

Abstract

A study was conducted to determine the effect of a one atmosphere uniform glow discharge plasma (OAUGDP) on inactivation of nalidixic acid resistant *Escherichia coli* O157:H7 and *Salmonella*, and *Listeria monocytogenes* on apples, cantaloupe, and lettuce, respectively, and culture media [tryptic soy agar (TSA) + 50 ppm nalidixic acid (TSAN) for *E. coli* O157:H7 and *Salmonella*; TSA for *L. monocytogenes*). A mixture of cultured test organisms was washed, suspended in phosphate buffer and spot inoculated onto produce or culture media (6 log CFU/sample). Inoculated produce or culture media (samples) were exposed inside a chamber affixed to the OAGUDP blower unit, operated at a power of 9 kV and a frequency of 6 kHz. This configuration allows the sample to be placed outside of the plasma generation unit, while allowing airflow to carry the antimicrobial active species, including ozone and nitric oxide, onto the sample. Cantaloupe and lettuce samples were exposed for 1, 3, and 5 min, while apple samples were exposed for 30 sec, 1, and 2 min. All culture media was exposed for 10, 30 sec and 1 min. After exposure, samples were pummeled in 0.1% peptone water containing 2% Tween 80, serially diluted, and plated in duplicate onto TSAN or TSA (both considered as non-selective) and selective media, and incubated as follows: *E. coli* O157:H7 (TSAN, modified EMB) and *Salmonella* (TSAN; XLT4), 48 hr, 37°C; *L. monocytogenes* (TSA and MOX); 48 hr, 32°C).

Generally, survival curves for all pathogens as indicated by recovery on non-selective and selective media followed a biphasic pattern. Specifically, a sharp decrease in

populations typically was observed after plasma treatment for the initial exposure time, followed by a decline in inactivation rate, or tailing effect, observed during after longer treatment times. This biphasic pattern was observed using both recovery media, although recovery on the selective medium was typically poorer than recovery on the non-selective medium. *L. monocytogenes* on lettuce did not follow this typical inactivation pattern when exposed to OAUGDP, although the biphasic inactivation pattern was observed when the organism was exposed to plasma on TSA. An approximate 3-log reduction was seen with *E. coli* O157:H7 on apples after exposure to plasma for two min, and similar levels of reduction were achieved with *Salmonella* and *L. monocytogenes* on cantaloupe rinds and lettuce, respectively, after three min of exposure to plasma. *L. monocytogenes* proved to be the slightly more sensitive to plasma treatment than *E. coli* O157:H7 and *Salmonella*. Populations of *L. monocytogenes* were reduced to undetectable levels and barely detected when lettuce and TSA, respectively, were exposed to plasma for 5 min. *E. coli* O157:H7 and *Salmonella* populations were never reduced to below 1 log CFU/sample. In all cases, substantially longer exposure times were required for reduction of pathogens exposed on produce as compared with exposure on culture media.

Differences in recovery of pathogens on selective and non-selective media revealed that substantial portions of the surviving populations of all pathogens were sublethally injured by plasma treatment. Generally, injury development was greater when pathogens were exposed to plasma on produce than on culture media, with the exception that *L. monocytogenes* underwent greater injury when exposed on culture media**.**

Plasma treatment of produce surfaces has the potential to be used in many areas of

the food processing industry. The process can reduce bacterial populations by several log units with a few minutes without causing physical damage exposed produce. By combining plasma treatment with other antimicrobial treatments, the ability to obtain safe and wholesome produce may be improved.

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Chapter I

Literature Review

Non-thermal inactivation of foodborne pathogens

Food safety is a major concern of every sector of the food industry. Scientists continue to evaluate new and innovative means of inhibiting or destroying pathogenic microorganisms in foods. With the preservation of fresh food quality being of utmost importance, process applications must not only be affective at destroying microorganisms, but they should not cause unacceptable changes in physical and organoleptic properties of food. Traditional methods of inactivating or killing pathogens in foods have relied on thermal processes. The rise in demand for fresh or minimally processed fruits and vegetables has spurred research to develop novel, non-thermal methods of reducing microbial populations without sacrificing quality. Non-thermal processes such as high hydrostatic pressure, irradiation, and pulsed electric fields have been studied as possible alternatives (Knorr, 2002).

High pressure processing (HPP) has been widely studied as a means of inhibiting microorganisms since the 1980's (Hoover, 2002). The antimicrobial action of HPP is related primarily to its effect on the cell envelope. Cellular morphology is altered by pressure, and cell division slows with increased pressure application (Hoover, 2002). Pressurized membranes show increased permeabilities due to protein membrane

denaturation that reduces amino acid uptake (Paul and Morita, 1971). When studying scanning electron micrographs of bacterial cells that were exposed to pressures >500 MPa, physical disruption of the cell envelope is common. However, with pressures <500 MPa, it is possible to view internal cellular damage with transmission electron microscopy with no obvious external cell damage (Hoover, 2002). Aleman and others (1994) showed that the shelf-life of fresh-cut pineapple could be extended by applying 340 MPa for 15 min. This process works very well as a means of reducing bacterial populations on fruit. However, HPP may not denature browning enzymes, and will require an additional process if this is a concern. When Arroyo and others (1997) studied the effects of HPP on inoculated lettuce and tomatoes, it was found that pressures of 300-350 MPa for 10 min reduced Gram-negative and Gram-positive bacteria by at least one log. During processing, the tomato skin began to peel away and browning occured in lettuce, which would make HPP unacceptable for processing these fresh products.

Pulses of high-voltage electric fields (PEF) are effective at inactivating a wide range of microorganisms (Zhang and others, 1994; Martin and others, 1997; Qin and others, 1996). The cell membrane of a microorganism is adversely affected when an electric field of high strength (kV) is applied at short-time pulses (μs) to aqueous suspensions (Hulsheger and others, 1983). Treatment of raw skim milk (2% fat) with PEF caused no significant changes in the chemical and physical properties of milk and extended the shelf-life two weeks (Zhang and others, 1995). Mechanisms of microbial inactivation that have been offered for PEF include electric breakdown, ionic punch-through effect, and electroporation of cell membranes (Barbosa-Canovas and others,

1999). Microbial inactivation occurs through irreversible electropermeabilization of cell membranes due to channeling and electroporation during exposure to PEF. However, this technology is limited to liquid media as a suitable means of inactivating microorganisms.

Irradiation refers to any process involving the application of ionizing radiation (Mendonca, 2002). This includes x-rays, gamma rays, or machine generated electron beams, but gamma rays are used most commonly in the processing of foods due to their excellent penetration characteristics. While the ionizing radiation provided by e-beams is in the form of electrons, in the case of X-rays and gamma rays, it is provided by photons. The latter have no mass and are thus able to penetrate deeper into materials. Electrons, on the other hand, have a small mass, and are characterized by more limited penetration. Microbial inactivation occurs due to hydrogen and hydroxyl free radical interactions with the bacterial DNA and the cell envelope, and the direct removal of electrons from the DNA resulting in irreversible damage (Mendonca, 2002). Low doses (<1 kGy) can be used to destroy insects and other pests in fruits and vegetables. Doses of 1-10 kGy (medium dose) will achieve pasteurization in most foods, and high doses of >10 kGy can achieve commercial sterilization. *Salmonella* has been found to be most resistant to irradiation among all foodborne, Gram-negative, pathogenic bacteria. Doses of 1.5-5.0 kGy have been found to be effective in reducing *Salmonella* populations (Mendonca, 2002). Although irradiation is effective in reducing microbial populations in foods, consumer acceptance is still a concern. Resurreccion and others (1995) found in a survey of consumers that perception of irradiation was improving, but many consumers are still concerned about the safety of irradiated products. The high cost of implementing one of

these systems also limits its use in the food industry (Mendonca, 2002).

Bactericidal effects of plasma

Neutral particles, electrons and positively charged atoms and molecules comprise plasma, the fourth state of matter (Madea and others, 2003). When a gas passes through plasma, the gas becomes excited, ionized, or dissociated by electron collision (Ben Gadri and others, 2000). This leads to the formation of active species such as atomic oxygen, ozone, and free radicals (ie., hydroxyl, superoxide, and nitrogen oxides) (Laroussi and Leipold, 2003). These reactive species have been shown to have antimicrobial activity as a result of their interaction with the surface of the microorganism (Ben Gadri and others, 2000; Guzel-Seydim and others, 2003; Kelly-Wintenberg and others, 1999; Larousi and Leipold, 2003).

Kelly-Wintenberg and others (1999), using a one atmosphere uniform glow discharge plasma (OAUGDP), reported plasma membrane damage of *Escherichia coli* K12, evident by an increase in optical density of the culture suspension after plasma exposure, indicating the leakage of proteins and/or nucleic acids from the plasma membrane. This theory was supported by the visual appearance of *E. coli* K12 after 30 sec of plasma exposure. The cell wall and plasma membrane were disrupted, which caused the leakage of intracellular constituents across the cellular envelope (Kelly-Wintenberg and others, 1999). When the OAUGDP is operated in air, the relatively long duration of the uniform plasma during each half-cycle of the radio frequency (RF), compared to dielectric barrier discharges (10-100 microseconds compared to nanoseconds), allows complex plasma chemistry to occur. This complex chemistry results

in the production of nitrogen oxides, ozone, species in excited and metastable states, and atomic oxygen. The killing mechanism responsible for sterilization in past studies is consistent with destructive oxidation of microbial constituents, and atomic oxygen appears to be one of the principal oxidizing agents (Kelly-Wintenberg and others, 1999). Atomic oxygen is potentially a very effective sterilizing agent. Its chemical rate constant for oxidation at room temperature is about 10^6 times that of ordinary molecular oxygen

Reactive oxygen species (ROS), such as oxygen radicals, most effect the membrane lipids by unsaturated fatty acid peroxide formation (Montie and others, 2000). Oxidation of amino acids that and nucleic acids may also cause changes that result in microbial death or injury. Double bonds of unsaturated lipids are particularly vulnerable to ozone attack (Guzel-Seydim and others, 2003). Membrane lipids are thought to be more affected by ROS due to their location along the surface of the bacterial cell, which allows them to be bombarded by these strong oxidizing agents (Montie and others, 2000).

Differences among microorganisms

The extent of differences in plasma destruction between Gram-positive and Gram-negative bacteria is due to the structure of their cell walls. The Gram-positive cell wall has a thick peptidoglycan layer that is 20 to 80 nm thick (Talaro and Talaro, 1999). This peptidoglycan layer is composed to acidic polysaccharides, such as techoic and lipotechoic acids, that are tightly bound together. These acidic polysaccharides are responsible for the positive charge on the cell surface. The periplasmic space between the peptidoglycan layer and the cell wall varies with bacteria.

The Gram-negative cell wall is slightly more complex because it contains an outer membrane, a thin peptidoglycan layer, and periplasmic space between the peptidoglycan layer and cell membrane (Talaro and Talaro, 1999). The outer membrane is similar to the cell membrane in some ways because it is composed of a bilayer made up of unique polysaccharides and proteins. The outermost layer contains lipopolysaccharide (LPS), which is a polysaccharide fragment integrated into membrane lipids. The innermost layer is composed of a lipid layer anchored by proteins to the thin peptidoglycan layer below. The outer membrane acts as a barrier that only allows relatively small particles to penetrate. Special membrane channels composed of porin proteins allow molecules to transverse the entire width of the outer membrane. The peptidoglycan layer still gives a rigid structure to the cells, but it is only one to three nm thick. This thin layer allows for more flexibility and makes the Gram-negative bacteria more sensitive to lysis.

Cell physical damage has been documented as the lethal effect of the OAUGDP, and physical damage to the cell envelope was greater for *E. coli* O157:H7 than *S. aureus* (Montie and others, 2000). Release of macromolecules, and thus death of the cells caused by cytoplasmic membrane alteration by the OAUGDP, was evidenced by an increase in absorbence when examined spectrophotometrically (Montie and others, 2000). Additionally, transmission electron micrographs clearly show ruptured *E. coli* cells after 30 sec exposure to the OAUGDP. *S. aureus* also exhibited macromolecular leakage, but to a lesser extent than *E. coli* (Montie and others, 2000).

Members of the Gram-positive *Bacillus* and *Clostridium* spp., as well as some others, have the ability to form spores as a protective measure against unfavorable

conditions. Resistance of spores to sterilization processes is well documented and has been extensively studied (Setlow and Johnson, 1997). Kelly-Wintenberg and others (1998) showed a necessity for longer OAUGDP exposure times to destroy bacterial spores than vegetative cells. *Bacillus* spp. are aerobic bacteria that are capable of spore formation, while *Clostridium* spp. are anaerobic, and thus, are sensitive to oxygen. Aerobic bacteria produce enzymes, such as superoxide dismutase and catalase, that aid in neutralizing toxic forms of oxygen (e.g., superoxide free radicals and hydrogen peroxide). Obligate anaerobes, such as *Clostridium* spp., do not produce superoxide dismutase or catalase (Talaro and Talaro, 1999), and, although they produce spores, anaerobic sporeformers could be more sensitive to the OAUGDP than *Bacillus* because of the toxic oxygen species produced.

Glow discharge plasma

The uniform glow discharge plasma can be created at atmospheric pressure. However, there are other types of plasma, including those generated by corona discharge, dielectric barrier discharge, arcjets, and inductive plasma torches (Ben Gadri and others, 2000). Corona discharges produce active species non-uniformly and at levels too low for many applications, but this plasma configuration is the primary method used for commercial generation of ozone (Guzel-Seydim and others, 2003). Restaino and others (1995) studied the antimicrobial effects of ozonated water produced by a corona discharge. It was determined that a wide range of foodborne pathogens, including Gram-positive and Gram-negative bacteria, as well as some yeasts, were inactivated by exposure to gaseous ozone in water.

Dielectric barrier discharges have a very short plasma duration that does not allow development of the full sequence of chemical reactions necessary for the formation of reactive species. Castro and others (2003) conducted preliminary studies using a dielectric barrier glow discharge to in-line waste water treatment. The active species that were produced were sufficiently concentrated to interact quickly with water that was passing through at a rate of 2 mL/s, and the system was able to reduce microbial populations by 90% compared to the control (Castro and others, 2003).

Arcjets and inductive plasma torches heat the neutral gas and active species to temperatures in excess of 5,000 K. Their high power density (tens to hundreds of watts per cm^3) can damage many heat sensitive materials, such as fresh produce (Kelly-Wintenberg and others, 1999).

One Atmosphere Uniform Glow Discharge Plasma (OAUGDP)

Until recently, glow discharge plasmas had to be formed in a vacuum at pressures below 10 torr (Ben Gadri and others, 2000). The cost of the needed vacuum equipment greatly reduced the feasible application of this process. The One Atmosphere Uniform Glow Discharge Plasma (OAUGDP) is a novel, atmospheric, RF plasma, which can be generated over large areas and in large volumes. The primary feature that distinguishes the OAUGDP from other RF plasmas is its efficient ability to create a uniform glow discharge in air and other gases at atmospheric pressure, ambient temperature, without a vacuum system, and without a requirement for batch processing of samples.

The OAUGDP was made possible by the development of an ion trapping mechanism (Roth, 1995). The principle behind this mechanism relies on applying the

appropriate radio frequency that causes ions to oscillate between two electrode plates without reaching either boundary (Roth, 1995). The trapping of these ions creates the characteristic uniform glow. The OAUGDP is a non-thermal RF plasma with time-resolved characteristics of a classical low pressure DC normal glow discharge that operates on displacement currents and provides an air plasma that creates high fluxes of energetic active species at one atmosphere. Glow discharges, including the OAUGDP, are very energy efficient while maintaining themselves in an ionized state. This technology allows for a uniform glow discharge plasma, which most uniformly distributes antimicrobial active species across a surface for sterilization in the presence of any gas. Glow discharges operate at low to moderate areal power densities, typically less than one W/cm^2 (Roth and others, 2000). These properties make this technology ideal for processing of heat-sensitive materials.

The OAUGDP was first designed as a parallel plate reactor (MOD IV) system (Fig 1, all figures are located in appendix) (Ben Gadri and others, 2000). The parallel plate reactor is a stainless steel enclosure with interior dimensions of 40 x 35 x 35 cm. The water-cooled electrodes are covered with a dielectric to inhibit arcing. A characteristic pair of rectangular electrodes has dimensions of 18 x 15 cm. In this reactor, the parallel plates are energized with an oscillating RF voltage, typically in the range from six to seven kilohertz. The MOD IV reactor could be operated in the presence of argon, helium, or air and required an electric field of 8.5 kV/cm to initiate the glow plasma (Ben Gadri and others, 2000). However, samples were required to be placed directly into the plasma, and the gap between electrodes was only approximately 1.5 cm.

Gram-positive and Gram-negative bacterial populations were reduced when exposed on glass and polypropylene fabric in the MOD IV unit, and the inactivation curve, in general, was biphasic. This biphasic curve was characterized by an initial, rapid decrease in bacterial populations followed by a tailing effect during the middle exposure times, and finally a second, sharp decline in bacterial populations after longer exposures (Montie and others, 2000; Roth and others, 2000). However, the small electrode gap distance greatly hampered the size of sample that could be exposed.

A remote exposure reactor (RER; MOD V) (Fig 2) allowed for larger samples to be exposed to the exhaust from plasma generated from panels inside the reactor (Roth and others, 2000). The remote exposure reactor was constructed to remove active species from the site of generation between two electrodes to a remote chamber at least 20 cm from the nearest plasma, where the samples to be sterilized are exposed without exposure to such direct effects such as UV radiation, ion bombardment, or strong electric fields. A serpentine airflow passed over the panels generating plasma and carried the antimicrobial active species with the exhaust onto the sample present in the chamber below. In this configuration, the exhaust can be recirculated back through the RER, which would concentrate species that were sustained long enough to pass through the loop at least once (Roth and others, 2000). However, the panels of this reactor could not be cooled and caused the temperature of the exhaust to rise very quickly; so samples could only be exposed for 25 sec periods to prevent their (parallel plate) temperature from rising above 49°C.

When compared to the MOD IV reactor, the RER was also capable of inactivating

pathogens at a faster rate during the early stages of exposure but did not have the biphasic inactivation curve that was seen with the MOD IV reactor (Roth and others, 2000). To identify macromolecular characteristics of plasma treated vegetative cells, transmission electron microscopy was performed. Within 5 sec of exposure, the outer membrane and plasma membrane of *E. coli* K12 cells are not distinguishable, and the pili originally covering the cells disappeared (Roth and others, 2000). In order to process heat sensitive materials in the RER, the exhaust should be kept as close to room temperature (25°C) as possible. Therefore, some means of cooling the plasma conduction plates must be derived to reduce exhaust temperature if continuous processing can be achieved (Roth and others, 2000).

A blower exposure unit was developed to address concerns for heat production (Fig 3). The blower unit produces plasma inside a tubular configuration that allows airflow to pass through carrying actives species with the exhaust. The plasma conduction surface is cooled by oil that is recirculated at 20°C. A radiator is also mounted onto the bottom of the blower unit that allowed the exhaust to be kept at approximately 22°C. This configuration allows for the control of the airflow as in the RER, but does not allow for the recirculation of exhaust. This system is the first developed that does not expose the sample inside a chamber, and special care must be made to ensure safe removal of exhaust. The blower configuration ideally allows for remote exposure of various sized samples, but research data are lacking on this latest reactor configuration.

Produce pathogen relationships

In the United States, foodborne pathogenic bacteria including *Salmonella, Listeria monocytogenes, E. coli* O157:H7, and *Campylobacter jejuni* are estimated to account for millions of cases of diarrheal diseases each year (USDA, Economic Research Service, 2003). With these illnesses come increased medical costs and loss of productivity and business. Several large outbreaks of foodborne illness associated with cantaloupe, lettuce, tomatoes, apple and orange juice, and many other small-scale outbreaks have been linked to fresh produce (Brackett, 1999).

Many pathogenic microorganisms can survive relatively harsh conditions of prolonged exposure in feces, soil, and water. Fruits and vegetables can become contaminated with pathogenic microorganisms at many points along the production line, including during growth in fields and orchards, harvesting, post-harvest handling, processing, and distribution (Beuchat, 1996). Contamination of produce with pathogenic bacteria can come from contaminated water, soil, and feces of wild and domesticated animals and humans. Harvesting equipment, produce containers, feces, wash and rinse water, and cross-contamination from other foods in the same storage area can all be sources of pathogenic bacterial contamination of fruits and vegetables (Beuchat, 1996).

The genus *Salmonella* is composed of over 2,700 serotypes. Every year, approximately 40,000 cases of salmonellosis are reported in the United States, and since many other cases are not diagnosed or reported, the actual number of infections may be twenty or more times greater (CDC, 1997). Animals and birds have been described as natural reservoirs for *Salmonella* (Beuchat, 1996), but *Samonella* spp. have been isolated

from many different types of fresh produce. Poultry and other meat products, eggs, and dairy products are typically the causes of salmonellosis outbreaks. However, several large outbreaks of salmonellosis have been attributed to fresh produce. In 1992 and 1993, two multi-state outbreaks of salmonellosis were caused by consumption of raw tomatoes (Hedberg, 1994). Melons have also been implicated in outbreaks of salmonellosis. A 1990 outbreak implicating cantaloupe contaminated with *S. chester* was estimated to affect 25,000 individuals, with two deaths resulting (Beuchat, 1996). The Centers for Disease Control and Prevention (1991) reported another outbreak caused by the consumption of cantaloupe that was contaminated with *S. poona*. This outbreak occurred across at least 25 states in the United States and 185 cases were confirmed. This outbreak was associated with cantaloupe that was consumed on a salad bar, which suggests that bacterial growth on cantaloupe occurred after cutting (Beuchat, 1996). Golden and others (1993) showed that *Salmonella* populations on the interior tissues of cantaloupe, watermelon, and honeydew melon could increase by 5 to 7 log units after 24 hr at 23°C. These data demonstrate that if the rind or cutting utensil used for slicing of melons is contaminated with *Salmonella* spp., transmission and proliferation of this pathogen on the edible flesh can occur.

E. coli O157:H7 is a member of the enterohemorrhagic *E. coli* (EHEC) and causes hemorrhagic colitis in humans (IFT 2000). *E. coli* O157:H7 illness symptoms generally include severe abdominal cramps and diarrhea (often times bloody), which can lead to death. In children under the age of five, infection can lead to a complication known as hemolytic uremic syndrome (HUS), resulting in red blood cell destruction, renal failure,

and central nervous system complications (Parsonett and Griffen 1993). There are an estimated 73,000 annual cases of *E. coli* O157:H7, leading to an estimated 2,100 hospitalizations in the United States (CDC 1993). *E. coli* O157:H7 can cause disease at a low infectious dose (10 - 100 cells). This can be attributed to the tolerance of this bacterium to low pH, which allows passage through the stomach and colonization in the intestinal tract (IFT 2000).

In October 1996, unpasteurized apple cider and juice were associated with two outbreaks of *E. coli* O157:H7. The first outbreak occurred in the Western United States and was associated with unpasteurized apple juice. This outbreak caused 66 human illnesses and one death (CDC 1996). The second outbreak occurred in the Northeastern United States and involved contaminated apple cider, which resulted in illness to 14 people and one case of HUS (CDC 1996).

Apple cider is commonly manufactured locally at small cider mills. The apples are crushed in presses and the cider is frequently unpasteurized. Although the exact mechanism for contamination is not known in these outbreaks, manure was suspected to have contaminated the apples used in cider making (CDC 1996). Apples that have already fallen from the tree are often used to make apple cider (Besser et al 1993), and if these apples come in contact with manure that is contaminated with pathogens, then they too can become contaminated. An outbreak of *E. coli* O157:H7 infections in 1991 occurred at a cider mill whose orchards served as an area for cattle (Besser et al 1993). The cattle grazed adjacent to the mill, and this resulted in manure inadvertently contacting the apples (Besser et al 1993).

In the United States, an estimated 2,500 persons become seriously ill with listeriosis, an infection of *L. monocytogenes,* each year, and of these 500 die (CDC, 2003). Although listeriosis is not as prevalent as infections from *Salmonella* and *E. coli* O157:H7, high mortality rates make *L. monocytogenes* a serious concern as a foodborne pathogen. *L. monocytogenes* is commonly found on plant vegetation due to contamination by feces, irrigation water, or humans (Jay 1996). Vegetables that are used for salads are a major means of spreading this pathogenic bacterium into the food supply (Beuchat, 1996). Sizmur and Walker (1988) found that 4 of 60 prepacked, ready-to-eat salads sold in the United Kingdom were positive for *L. monocytogenes*. These salads were composed of lettuce, cucumber, onion, cabbage, celery, carrots, leeks, watercress, and fennel, any of which may have been sources of contamination. Garcia-Gamino and others (1996) tested commercial, ready-to-eat mixed salad and determined that out of a total of 70 control (noninoculated) samples, 21 (30%) were contaminated with *L. monocytogenes*. A study of salad inoculated with 10^3 cfu/g *L. monocytogenes* showed that the organism increased less than ten-fold during 300 h of storage (Garcia Gamino and others 1996).

Since *L. monocytogenes, E. coli* O157:H7, and *Salmonella* spp. have all been linked with foodborne disease outbreaks related to consumption of produce, they all serve as model pathogens for testing the efficacy of novel, non-thermal antimicrobial processing of fruits and vegetables. If a process is to become widely employed in the food industry, it must first be validated in food production and processing systems. Many times, an antimicrobial process may be very effective *in vitro* and have must less efficacy

when evaluated with food. Since the OAUGDP has not been extensively tested in a food system, research must be conducted in this area.

Chapter II

Materials and Methods

Preparation of inoculum

E. coli O157:H7

Four strains of nalidixic acid resistant *E. coli* O157:H7 including, H1730 (lettuce-associated outbreak), F4546 (alfalfa sprout-associated outbreak), E0019 (beef-associated outbreak), and 932 (human feces), held in the University of Tennessee Food Microbiology laboratory culture collection, were used to inoculate unwaxed red delicious apples. Each test strain was cultured in tryptic soy broth (TSB; Difco Becton Dickinson Microbiology Systems; Sparks, MD) supplemented with 50 ppm nalidixic acid (Fisher Scientific; Pittsburgh, PA) (TSBN) for 24 hr at 37°C. Cultures were transferred a minimum of three times at 24 hr intervals before use.

The four test strains were combined to yield a mixed culture contained equal proportions of each test strain (20 mL). Cells were harvested by centrifugation at 8000 x g for 12 min, followed by a resuspension in 20 mL of 0.1 M phosphate buffer (PB; Becton Dickinson Microbiology Systems; Sparks, MD). This procedure was repeated two times. The final resuspended, mixed culture was diluted in PB to achieve a population of approximately 7 log CFU/mL.

Salmonella

Five serovars of nalidixic acid resistant *Salmonella*, *S.* Agona (alfalfa sprout-associated outbreak), *S.* Montevideo (tomato-associated outbreak), *S.* Gaminara (orange juice-associated outbreak), *S.* Michigan (cantaloupe-associated outbreak), and *S.* Baildon (lettuce and tomato-associated outbreak) held in the University of Tennessee Food Microbiology laboratory culture collection were used to inoculate cantaloupe rinds. Each test strain was cultured in TSBN for 24 hr at 37°C, with a minimum of three 24-hr transfers before use. The five serovars were combined to yield a mixed culture of equal proportions of each test strain (25 mL), and the cells were harvested as described for *E. coli* O157:H7.

L. monocytogenes

Five strains of *L. monocytogenes*, 101 (hard salami isolate), 108 (hard salami isolate), 310 (goat cheese isolate associated with spontaneous abortion), Scott A (clinical isolate), and V7 (raw milk isolate) held in the UT Food Microbiology laboratory culture collection were used to inoculate lettuce leaves. Each test strain was cultured in TSB for 24 hr at 32°C, with a minimum of three 24-hr transfers before inoculation. The five test strains were combined to yield a mixed culture of equal proportions of each test strain (25 mL). The 5-strain culture suspension was washed and harvested as described for *E. coli* O157:H7

Inoculation of media

E. coli O157:H7 and *Salmonella* were inoculated onto tryptic soy agar (TSA; Difco) containing 50 ppm nalidixic acid (TSAN). *L. monocytogenes* was inoculated onto TSA. Media were sterilized and poured (~20 mL) into 100 mm diameter Petri dishes 24 hr before use. Media were surface inoculated under a class II biological safety cabinet (Fisher Scientific; Pittsburgh, PA) with 10 µL of culture suspension in 10 spots to achieve a total inoculum volume of 100 µL. Inocula were allowed to absorb into covered media for two hr under biological safety cabinet, and the plates were stored at 4°C for 22 hr Inoculated media contained approximately 6 log CFU of *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes* per plate.

Inoculation of produce

Choice of inoculum

Test produce were inoculated with the pathogen most associated with each type of produce or recognized as having been linked to outbreaks of illness with the type of inoculated produce. As such, apples, cantaloupe, and lettuce were inocualted with *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*, respectively, as described below.

E. coli *O157:H7 inoculated onto apples*

Unwaxed red delicious apples were obtained from a local apple grower. Apples were washed in deionized water, and the stem ends were cut off to a thickness of 3 cm and placed peeling side up in sterile 100 mm Petri dishes. Apple end sections were spot inoculated under a class II biological safety cabinet with 10-µL aliquots of inoculum in 10

spots on peeling. Dishes were covered, the inoculum was allowed to dry for two hours under the biological safety cabinet, and the Petri dishes containing inoculated apple sections were stored at 4°C for 22 hr. Each apple section contained approximately 6 log CFU of *E. coli* O157:H7.

Salmonella *inoculated onto cantaloupe*

Cantaloupe melons were obtained from a local grocery store. Cantaloupe rinds were removed, trimmed to remove the edible mesocarp, and cut into 25 cm^2 sections (5 cm x 5 cm). Rind sections were exposed to low-pressure steam (180°C) in a steam cabinet for 30 sec to reduce populations of indigenous microflora and placed in sterile 100 mm diameter Petri dishes. Rind sections were spot inoculated under a class II biological safety cabinet with 10-µL aliquots of inoculum in 10 spots on the outer rind. The inoculum was allowed to dry for one hr under the biological safety cabinet, and the dishes were covered and stored at 4°C for 22 hr. The *Salmonella* populations on rinds was approximately 6 log CFU/ 25cm^2.

L. monocytogenes *inoculated onto lettuce*

Iceberg lettuce was obtained from a local grocery store. The outer three leaves of lettuce were removed and discarded, and the remaining head of lettuce was rinsed with deionized water. Lettuce leaves were removed, cut into 25 cm^2 sections (5 cm x 5 cm) and placed in sterile 100 mm diameter Petri dishes. The lettuce leaf was spot inoculated under a class II biological safety cabinet with 10 µL aliquots of inoculum in 10 spots. The inoculum was allowed to dry on the lettuce samples for one hr under the biological

safety cabinet, dishes were covered, and then stored at 4°C for 22 hr. Inoculated lettuce contained approximately 6 log CFU of *L. monocytogenes*/ 25 cm^2.

Exposure of samples to a OAUGDP

An OAUGDP blower exposure unit (Fig. 3) was utilized to expose samples to antimicrobial active species produced in the OAUGDP exhaust. This unit produced plasma inside a tubular configuration that allowed airflow to pass through carrying actives species with the exhaust at a rate of 70-90 cubic feet per minute. The plasma conduction surface was cooled by oil that was recirculated internally at 20°C. A cold water radiator was mounted onto the bottom of the blower unit that allowed the exhaust to be kept at approximately 25°C. The uniform glow discharge plasma was produced at a voltage of 9.0 kV, a frequency of 6.0 kHz, and a gap distance of approximately 1.5 mm was maintained between electrodes. Inoculated samples were kept at 4°C during transport to and from the OAUGDP reactor.

Samples were exposed in the sterile plastic Petri dish, in which they were stored, with lids removed during exposure. Three samples were exposed to plasma for each of three exposure times in a rectangular chamber mounted onto the radiator. The chamber was open on each end to allow for exhaust flow-through while concentrating the antimicrobial active species in the OAUGDP exhaust. The configuration of the treatment chamber was 15.2 cm x 15.2 cm x 30.4 cm (height x width x length). The Petri dish in which the sample was held was placed in the chamber such that the surface of the media or produce (see Fig. 4) was 11.4 cm from the plasma and 5.0 cm from the top of the

sample chamber.

Enumeration of bacteria

E. coli *O157:H7 on TSAN and apples*

Inoculated and exposed TSAN was aseptically removed from Petri dishes using a sterile spatula and placed in sterile filter stomacher bag (Fisher; Pittsburgh, PA) with 100 mL of 0.1% peptone water (PW; Difco; Sparks, MD) containing 2% Tween 80 (ICN Biomedical, Inc; Aurora, OH). Treated apple sections were aseptically removed from Petri dishes and placed into sterile filter stomacher bags with 100 mL PW containing 2% Tween 80. Samples were pummeled in a stomacher-blender (Stomacher 400: Seward; England) for two minutes at 230 rpm, allowed to rest for one minute, and pummeled again for two minutes. Pummeled suspensions were serially diluted in PW and surface plated on TSAN and modified eosin methylene blue agar (MEMB; Clavero and Beuchat, 1995) in duplicate using a spiral plater (Don Whitley Scientific Limited**;** Yorkshire**,** England). Plates were incubated for 48 hr at 37°C before enumeration of *E. coli* O157:H7 using a Protocol automatic plate counter (Synoptics Limited; Cambridge, UK). Five to 21 presumptive positive colonies from one TSAN plate were steaked onto MEMB followed by incubation at 37°C for 48 hr. Typical *E. coli* O157:H7 colonies on MEMB were confirmed using the dry spot *E. coli* O157 latex test (Oxoid Limited; Hampshire, England).

Salmonella *on TSAN and cantaloupe*

Exposed TSAN and cantaloupe rind sections were aseptically removed from Petri dishes and placed in sterile filter stomacher bags with 100 mL PW. Samples were pummeled in a stomacher-blender for two minutes at 230 rpm, allowed to rest for one minute, and pummeled again for two minutes. Suspensions were serially diluted in PW and spiral plated onto TSAN and xylose lysine tergitol 4 agar (XLT4; Difco Becton Dickinson Microbiology Systems; Sparks, MD) in duplicate. Plates were incubated for 48 hr at 37°C before enumeration of *Salmonella* using a Protocol automatic plate counter. Five to 14 of the presumptive positive colonies from TSAN were streaked onto XLT4 agar followed by incubation at 37°C for 48 hr. Typical *Salmonella* colonies on XLT4 agar were considered positive without further conformation.

L. monocytogenes *on TSA and lettuce*

Exposed TSA and lettuce sections were aseptically removed from Petri dishes and placed in sterile filter stomacher bags with 100 mL PW containing 2% Tween 80. Samples were pummeled in a stomacher-blender for two minutes at 230 rpm, allowed to rest for one minute, and pummeled again for two minutes. Suspensions were serially diluted in PW and spiral plated onto TSA and modified Oxford medium (MOX; Difco) in duplicate. Plates were incubated for 48 hr at 32°C before enumeration of *L. monocytogenes* colonies using a Protocol automatic plate counter. Five to 16 presumptive positive *L. monocytogenes* colonies from TSA were streaked onto MOX, followed by incubation at 32°C for 48 hr. Typical black colonies on MOX were

considered positive for *L. monocytogenes* without further conformation.

Data analysis

All experiments were replicated three times. The statistical model consisted of a randomized block design, blocking on replication. Statistical analysis was conducted using the mixed models procedure (PROC MIXED) of SAS® 8.2 (SAS Institute Inc.; Cary, NC) and significance of factors set at $P<0.01$. Analysis of variance was used to determine statistical differences in survival of pathogens on different foods.

Chapter III

Results and Discussion

Overview

Survival of test pathogens exposed to the OAUGDP for various times on culture media and produce is shown in Figures 5-10. Generally, survival curves for all pathogens as indicated by recovery on non-selective and selective media followed a biphasic pattern. Specifically, a sharp decrease in populations typically was observed after plasma treatment for the initial exposure time, followed by a decline in inactivation rate, or tailing effect, observed during after longer treatment times. This biphasic pattern was observed using both recovery media, although recovery on the selective medium was typically poorer than recovery on the non-selective medium. A selective medium was used for each test pathogen to restrict growth of indigenous produce microflora that may grow on the non-selective media used (e.g., TSAN and TSA) and for evaluation of sublethal injury. While, technically, TSAN is at least partially selective for organisms tolerant to nalidixic acid (e.g., the nalidixic strains of *E. coli* O157:H7 used in this study), our preliminary evaluations have demonstrated that recovery of nalidixic acid resistant *E. coli* O157:H7 and *Salmonella* on TSAN is not different than recovery on TSA (data not shown). As such, TSAN was considered non-selective for the purposes of calculating

percent injury (see Table 1).

The need for using selective media for inhibiting growth of indigenous microflora was minimized by using nalidixic acid resistant strains of *E. coli* O157:H7 and *Salmonella*. Typically, indigenous microflora from apples and cantaloupe that grew on TSAN were substantially different in appearance (e.g., color, size, morphology) than the target pathogens, and were routinely identified as yeasts. However, populations of background microflora on cantaloupe rinds were often sufficiently high to impede enumeration of *Salmonella* on TSAN, despite the difference in appearance of background microflora. This difficulty was partially overcome by using the highly selective XLT4 for recovery, and also by the pre-treatment of cantaloupe rinds with steam, which served to "pasteurize" rinds and reduce populations of background microflora. Our test strains of *L. monocytogenes* were not resistant to nalidixic acid. However, background microflora on lettuce were almost completely eliminated from consideration by removal of the outermost leaves on lettuce heads before use.

Survival of pathogens on plasma treated culture media and produce

Survival of *E. coli* O157:H7 on TSAN exposed to the OAUGDP is shown in Figure 5. Based upon recovery on TSAN and MEMB, a rapid, initial decline in population (~3.5 log CFU decrease) was observed after plasma exposure of 0.2 min. The slope of the inactivation curve decreased substantially during longer exposure treatments, with an additional decrease in populations of *E. coli* O157:H7 of about 1 log after treatment for up to 1 min. Slight differences in recovery of *E. coli* O157:H7 on TSAN

and MEMB were observed during treatment. Differences in recovery on these selective and non-selective media indicates that portions of the surviving population of *E. coli* O157:H7 were sublethally injured by plasma treatment. After treatment for 1 min, 74.3% of the surviving *E. coli* population consisted of injured cells (Table 1). Because of these observed differences in recovery, true cell-death can be more appropriately assessed based upon recovery on TSAN, rather than MEMB. As such, unless otherwise qualified, from this point forward, inactivation of all pathogens tested in this study will be considered based upon recovery on the non-selective medium used (i.e., TSAN or TSA).

Figure 6 shows survival of *E. coli* O157:H7 on apple sections during treatment with plasma. Inactivation of *E. coli* O157:H7 on apples was similar to that observed for TSAN, although the initial reduction in population was not as great and the tailing effect was not as pronounced. Differences in recovery of *E. coli* O157:H7 from treated apples on TSAN and MEMB, however, were greater than differences in recovery from treated TSAN, particularly after the initial exposure time. After plasma treatment for 0.5 min, 95.7% of the E. coli O157:H7 population on apples was sublethally injured (Table 1). While injury development on plasma treated TSAN may have been initially as great as that observed with apples, it is possible that the more nutritious culture medium was more conducive to injury repair. While every effort was made to analyze test samples as quickly as possible after exposure to plasma, the time between exposure and microbial analysis was often as long as two hours, with exposed samples kept on ice until analyzed. After high pressure processing treatments of foods, it has been reported that plate counts of surviving bacterial populations can increase or decrease if analyzed after a significant

time delay between high pressure treatment and dilution and plating (FDA, 2000).

Salmonella was generally more sensitive to plasma treatment than *E. coli* O157:H7. While the initial reduction in *Salmonella* populations on exposed TSAN (~1.5 log CFU) was not as great, the overall survivor curve slope was substantially steeper, and the tailing effect was not as prominent (Figure 7). Additionally, sublethal injury was substantial, with injury levels reaching 94.8% after plasma exposure of TSAN for 0.5 min (Table 1). Initial plasma inactivation of *Salmonella* on cantaloupe rinds (Figure 8) was greater than on TSAN. A 2.7-log decrease in initial populations was observed after plasma treatment for 1 min, and the tailing effect after further treatments was quite pronounced.

Recovery of *Salmonella* on XLT4 was substantially poorer than recovery on TSAN, even from untreated cantaloupe rinds (~1.4-log difference; 0 min), indicating that XLT4 was at least partially inhibitory to *Salmonella*. While calculations show that sublethal injury of *Salmonella* on cantaloupe rinds was at least 81.7%, it is difficult to accurately make an assessment of injury because of the inherently poor recovery of *Salmonella* from cantaloupe on XLT4. Recovery of *Salmonella* on XLT4 from untreated TSAN was about 1.5 log lower than recovery on TSAN, again suggesting that the more nutritious culture medium facilitated better survival and/or minimized the effects of stress during storage. It should be noted that all test samples (culture media and produce) were held at 4°C for 22 hr after inoculation with pathogens. Certainly, during this cold storage period, test pathogens could have become stressed, particularly on the surfaces on relatively non-nutritious substrates (e.g., cantaloupe rinds), resulting in some death or the

inability of survivors to form colonies on recovery media (Park and Beuchat, 1999). Additionally, *Salmonella* cells may have become attached to the surface of cantaloupe rinds during storage, making it difficult to remove the cells for enumeration. Furthermore, the intricate webbed surface of cantaloupe rinds makes removal of *Salmonella* inherently more difficult than removal from the surface of culture media. Indeed, populations of *Salmonella* recovered on TSAN (non-selective) from untreated cantaloupe rinds was about 1.2 log CFU lower than recovery from untreated TSAN, indicating that *Salmonella* populations were either reduced during cold storage on cantaloupe rinds or became attached to rind surfaces.

L. monocytogenes proved to be more sensitive to plasma treatment than *E. coli* O157:H7 and *Salmonella*. Survival of *L. monocytogenes* during plasma treatment of inoculated TSA and lettuce is shown in Figures 9 and 10, respectively. *L. monocytogenes* populations were rapidly reduced on TSA after initial plasma treatment for 0.2 min, while only a 1.2-log reduction was observed on lettuce after the initial 1-min plasma treatment. Interestingly, however, the survivor curve tailing effect with *L. monocytogenes* was observed only with treated TSA, and not with treated lettuce. This observation is opposite of what was typically seen with *E. coli* and *Salmonella*. *L. monocytogenes* populations were eliminated on lettuce leaves after plasma treatment for 5 min, and detected at just above the detection limit on TSA treated for 1 min, demonstrating the sensitivity of *L. monocytogenes* to plasma as compared with that of *E. coli* O157:H7 and *Salmonella*. Additionally, plasma treatment caused substantial sublethal injury of *L. monocytogenes*; 97.7% injury was observed on TSA after plasma treatment for 0.5 min, and 93.4% injury

occurred on lettuce treated for 3 min.

Biphasic inactivation curves

Most of the inactivation curves seen in this study followed a biphasic pattern. Typically, after the initial exposure to plasma, inactivation curves decline sharply, followed by a tailing-off effect as exposure times increased. Tailing of survivors curves is a common occurrence in thermal processing studies. Among numerous studies of thermal inactivation of pathogens, tailing effects have been reported during thermal inactivation of *E. coli* O157:H7 (Juneja and others, 1997) and *L. monocytogenes* and *S. aureus* (Kamau and others, 1990). Additionally, biphasic inactivation patterns have been observed with chlorine treatment of *Yersinia enterocolitica* (Paz and others, 1993), ultraviolet treatment of various microorganisms (FDA,2000), and high pressure treatment of *Lactobacillus plantarum* (Ulmer, 2002). In all of these cases, tailing is attributed to a fraction of the population of test organisms that is resistant to the treatment applied. Alternatively, Humpheson and others (1998) suggested that biphasic thermal inactivation of *Salmonella* Enteritidis was due to *de novo* synthesis of heat shock proteins that provide protection during prolonged heat treatment. Another explanation for tailing effects suggests that a small number of cells in a population of bacteria could have incurred a simple, one-step mutation that is responsible for the differences in heat sensitivities between the two subpopulations (Buchanan and others, 1994; Moats and others, 1971).

Kelly-Wintenberg and others (1998) reported observation of two-slope (biphasic) survivor curves for *S. aureus* and *E. coli* on plasma-treated polypropylene fabrics.

However, they did not observe a tailing-type of biphasic inactivation, but rather, they observed an initial shouldering pattern followed by rapid inactivation after longer plasma treatments. They suggested that during the first phase of inactivation, the active species in plasma reacted with the outer membranes of bacterial cells, thereby resulting in damaging alterations. During prolonged plasma treatment, and once the damage to membranes is done, the reactive species penetrate deeper into the cells causing rapid inactivation during the second phase. In the present study, we observed an opposite, biphasic effect, in which rapid inactivation occurred after initial plasma treatment. It should be noted that the studies by Kelly-Wintenberg and others (1998) were conducted using a parallel plate reactor, while in our study, a remote, blower unit was used to concentrate active species distal to the cite of plasma formation. It is possible that concentration of active species from the blower unit used in the present study results in the more rapid initial inactivation that we observed, and that the tailing effect is due to a resistant fraction of the population.

Kayes (2000) also used a parallel plate plasma generator for inactivation of various foodborne pathogens on exposed agar media, but observed biphasic inactivation curves similar to those we observed. Kelly-Wintenberg and others (1998) used plasma to treat dry, non-nutritious substances, while our studies and those of Kayes (2000) used plasma to treat moist, microbially nutritious substrates. Perhaps the difference in composition of treated material (e.g., moisture content) accounts for our observed differences in biphasic inactivation patterns.

In addition to biphasic inactivation, multi-slope survivor curves for bacteria exposed to plasma have been reported. Roth and others (2000) demonstrated multi-slope

inactivation curves for *E. coli* and *S. aureus* on polypropylene fabrics treated with plasma in a remote exposure reactor, while similar inactivation patterns were observed by Kuzmichev and others (2001) for *Bacillus stearothermophilus* on stainless steel strips. Similarly, Laroussi and others (2000) reported the occurrence of multi-slope survivor curves for *E. coli* and *Pseudomonas aerugenosa* on nitrocellulose files directly exposed to plasma. These multi-slope inactivation curves were typically characterized by an initially steep decline, followed by a tailing region, a second steep decline, and finally, an ending tail region over the course of plasma exposure. While the mechanisms responsible for this multi-slope inactivation pattern has not been elucidated, the apparent differences in plasma tolerance among single populations of bacteria are interesting and warrant further study.

One manifestation of thermal damage in bacteria is the loss of membrane integrity and leakage of a intracellular components (Hurst, 1977). Solutes lost into the surrounding heating menstruum from disrupted cells can provide protection from heat inactivation of remaining, undamaged cells (Hurst and Hughes, 1978). Montie and others (2000) demonstrated that the plasma membranes of *E. coli* and *S. aureus* cells were damaged by plasma treatment, as evidenced by transmission electron microscopy. They further demonstrated leakage of macromolecular constituents in liquid media by spectrophotometric measurement of increases in absorbance after plasma treatment. As such, it seems plausible that an initial bombardment of bacterial cells resulting in rapid death and subsequent release of membrane constituents could lead to the release of highly oxidative materials into the treatment menstruum (e.g., membrane lipids, free fatty acids,

etc.). The presence of a large volume of oxidizable materials could serve as a "quenching" phase for the highly reactive, oxidizing species produced by plasma, thereby resulting in a decline in microbial inactivation. We did not determine if this occurs, but certainly, the prospect is likely and warrants further investigation.

Intrinsic parameters

When comparing plasma differences in plasma tolerance between Gram-positive (*L. monocytogenes)* and Gram-negative (*E. coli* O157:H7 and *Salmonella*) bacteria, our results did not reveal large differences. While *L. monocytogenes* was generally more sensitive to plasma treatment, its survival was not appreciably poorer than *E. coli* O157:H7 or *Salmonella*. Ben Gadri and others (2000) found that a 30 sec exposure of *E. coli* on polypropylene in a parallel plate reactor resulted in a breach of the cellular envelope followed by loss of cellular constituents. When *S. aureus* was treated with the same parameters as *E. coli* there was no apparent destruction of the cell envelope when visualized with transmission electron microscopy. However, spectrophotometric examination of treated *S. aureus* cultures revealed that cellular leakage did occur but was delayed due to decreased cell wall fragmentation. These results were expected to be caused by the difference in bacterial cell walls and the thick peptidoglycan layer that is associated with Gram-positive bacteria.

Effect of produce surface

The surfaces of produce generally proved to hinder inactivation of the various pathogens. When compared to inactivation curves obtained for pathogens inoculated onto

sterile agar, those obtained from produce required longer exposure times to obtain the same reduction levels. This is possibly due to the produce surface being very rough compared to the smooth surface of the agar. Certainly, the intricate webbing on the surface of cantaloupe rinds provides numerous sites for Salmonella to attach and potentially evade antimicrobial treatments.

Inactivation mechanisms of antimicrobial active species

The OAUGDP produces antimicrobial active species including ozone, monatomic oxygen, superoxide, hydroxyl radicals, and nitric oxide (Gadri and others, 2000). Atomic oxygen is a highly reactive oxygen species (ROS) that can react quickly since it can diffuse rapidly to alter cell membranes, proteins, and DNA. Other ROS, such as superoxide, hydroxyl radicals, and ozone that are produced can oxidize membrane lipids (causing unsaturated fatty acid formation), proteins, and nucleic acid oxidation (Montie and others, 2000). Montie and others (2000) theorized that the most vulnerable macromolecules affected by ROS were membrane lipids due to their location near the cell surface. This may also explain why Gram-positive bacteria have sometimes shown increased resistance to inactivation by antimicrobial active species produced by OAUGDP. The thick peptidoglycan layer in Gram-positive bacteria may serve as an additional support that would help retard leakage of cellular components.

Chapter IV

Summary

Biphasic inactivation curves were observed for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* inoculated to on apples, cantaloupe, and culture media (not observed with *L. monocytogenes* on lettuce) These biphasic curves can be described by initial steep declines in bacterial populations that occurred during the early plasma exposure times, followed by a tailing-off of bacterial inactivation with the later exposure times. Exposure times were greatly reduced when pathogens were exposed on culture media versus the various produce to the OAUGDP. The surfaces of apples, cantaloupe, and lettuce are more conducive to microbial attachment and also may provide physical barriers that protect against the antimicrobial active species produced by plasmas. The presence of indigenous microflora on produce surfaces may also reduce the effect of antimicrobial active species on pathogens present when compared to the otherwise sterile surface of culture media.

Variation in microbial inactivation may be partially attributed to operational parameters of the OAUGDP blower unit, which can vary the concentration of reactive oxygen species. Increased humidity is known to negatively impact the production of ozone. With increased humidity ozone more readily undergoes reactions that favor more

stable compounds. The OAUGDP blower unit used in this study was operated under ambient temperature, humidity, and pressure conditions. Since moisture in the air that was passed through the OAUGDP blower unit was not removed, high relative humidity could greatly impact the concentration of ozone in the OAUGDP exhaust. Much of this study was conducted during the summer and fall months, and indeed, Tennessee is certainly known for its high levels of humidity during these months.

Plasma technology will be limited in its application in the food industry due to the reactive oxygen species that are produced. Products which have high lipid content probably would be adversely affected by oxidation of lipid. Lipid oxidation results in the formation of hydroxy acids, keto acids, short chain fatty acids, and aldehydes that cause both off-flavors and odors. For these reasons, meat products such as hot dogs, raw beef, and luncheon meat would not be ideal substrates for treatment with plasma. Reactive oxygen species would also bombard antioxidants that are present in foods. This would cause the oxidation of vitamins C and E, which are naturally occurring antioxidants present in many foods, and loss of these naturally occurring products would be undesirable.

Decontamination of surfaces in food processing facilities may be a possible application of the OAUGDP blower configuration. The data collected from pathogens inoculated onto culture media indicate that microbial populations can be quickly inactivated on clean, flat surfaces. Furthermore, pathogen inactivation of up to several log units is readily accomplished within a few minutes on intricate, complex surfaces such as those of cantaloupe rinds. Biofilms are of concern to food processors, and application

plasma technology has possible applications for preventing biofilms from forming on processing surfaces.

Sublethal injury of test pathogens was observed on all products exposed to plasma, even after only short exposure periods. In addition to microbial destruction, advantage can be taken of injury in bacteria, which can serve as a "hurdle" during application of antimicrobial treatments to produce and other heat-sensitive food products. Injured cells that survive treatment with plasma likely would be more sensitive to additional antimicrobial treatments, and vice versa. By combining plasma treatment with other antimicrobial treatments, the ability to obtain safe and wholesome produce may be improved.

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Appendix

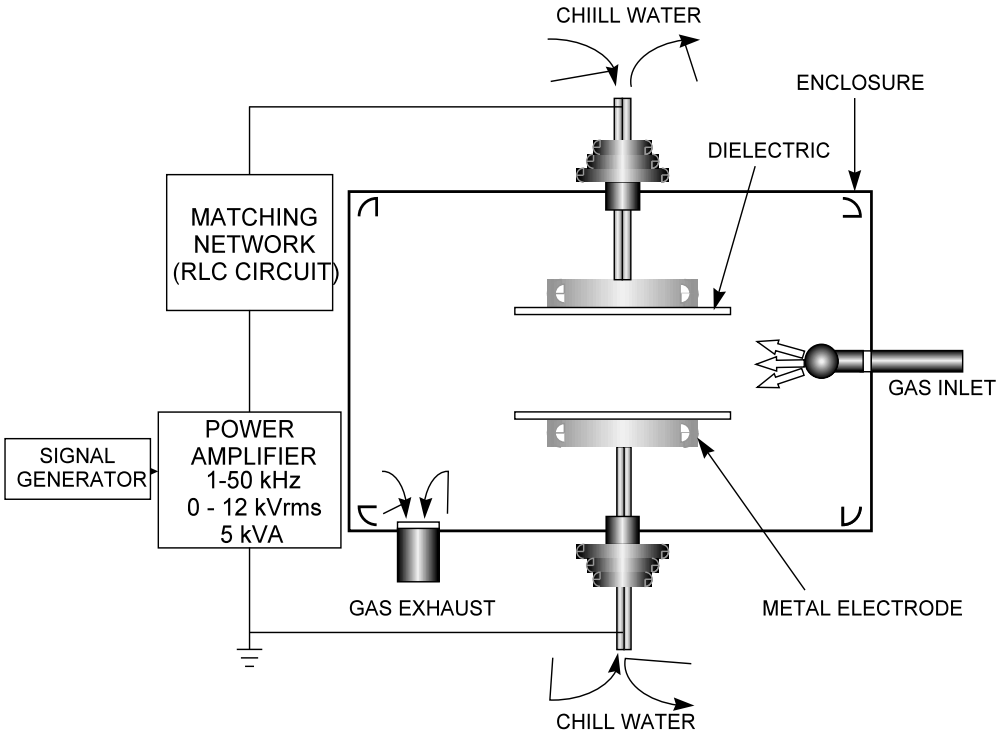

Figure 1. OAUGDP parallel plate reactor.

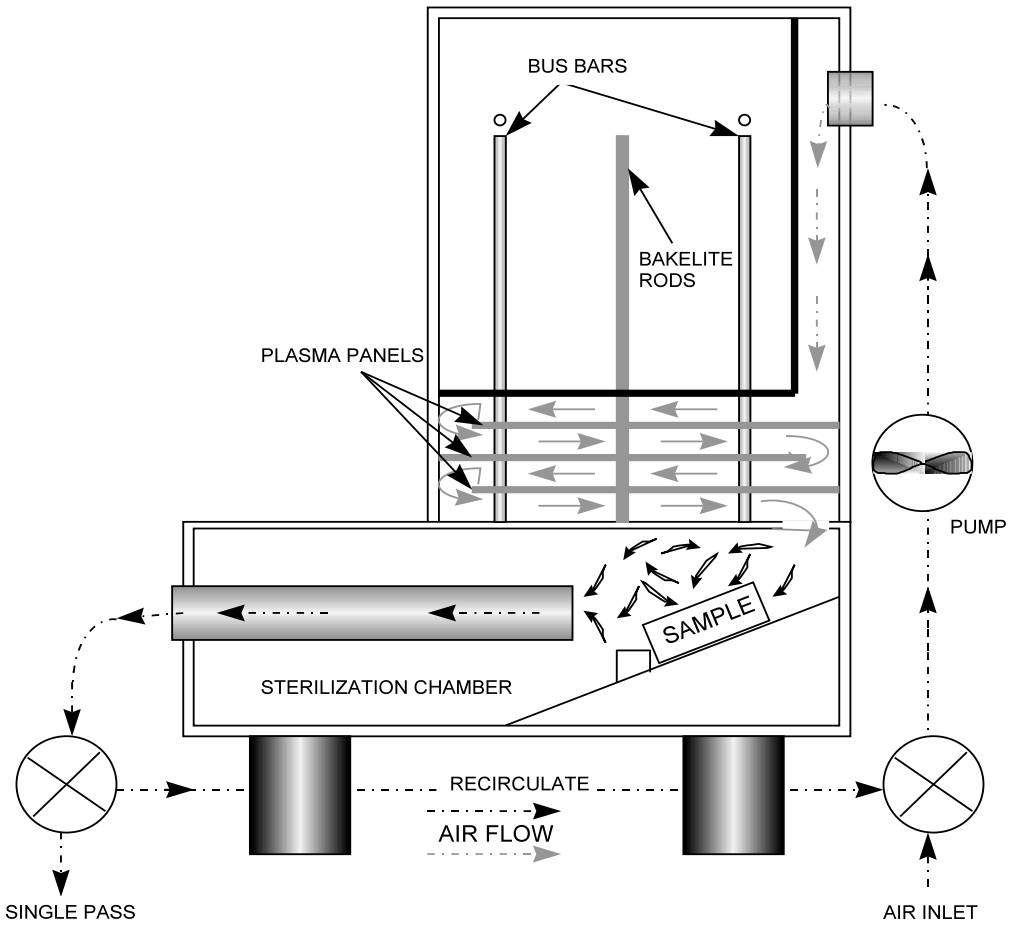

Figure 2. OAUGDP remote exposure reactor.

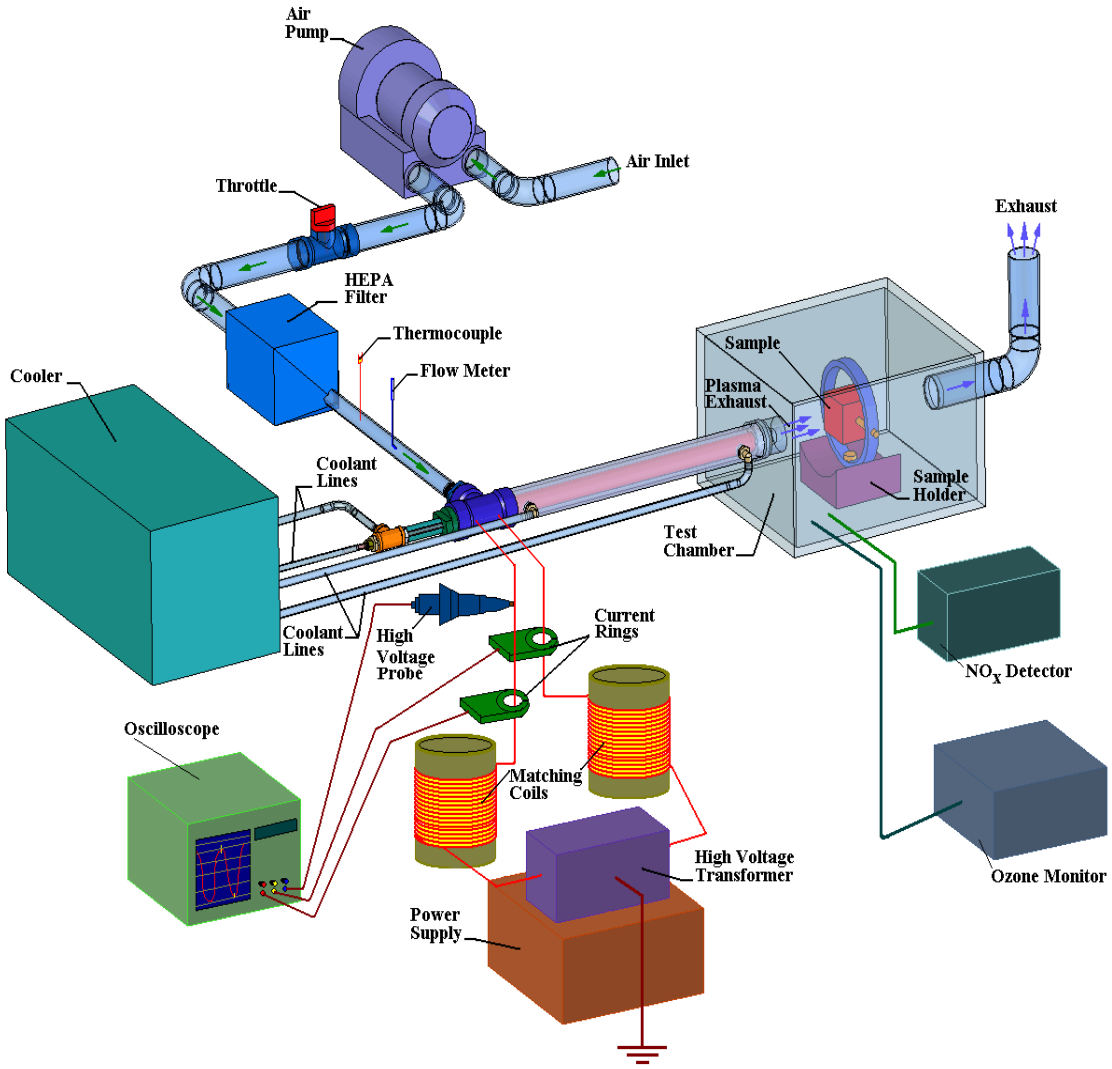

Figure 3. OAUGDP blower unit

schematic provided by atmospheric glow technologies.

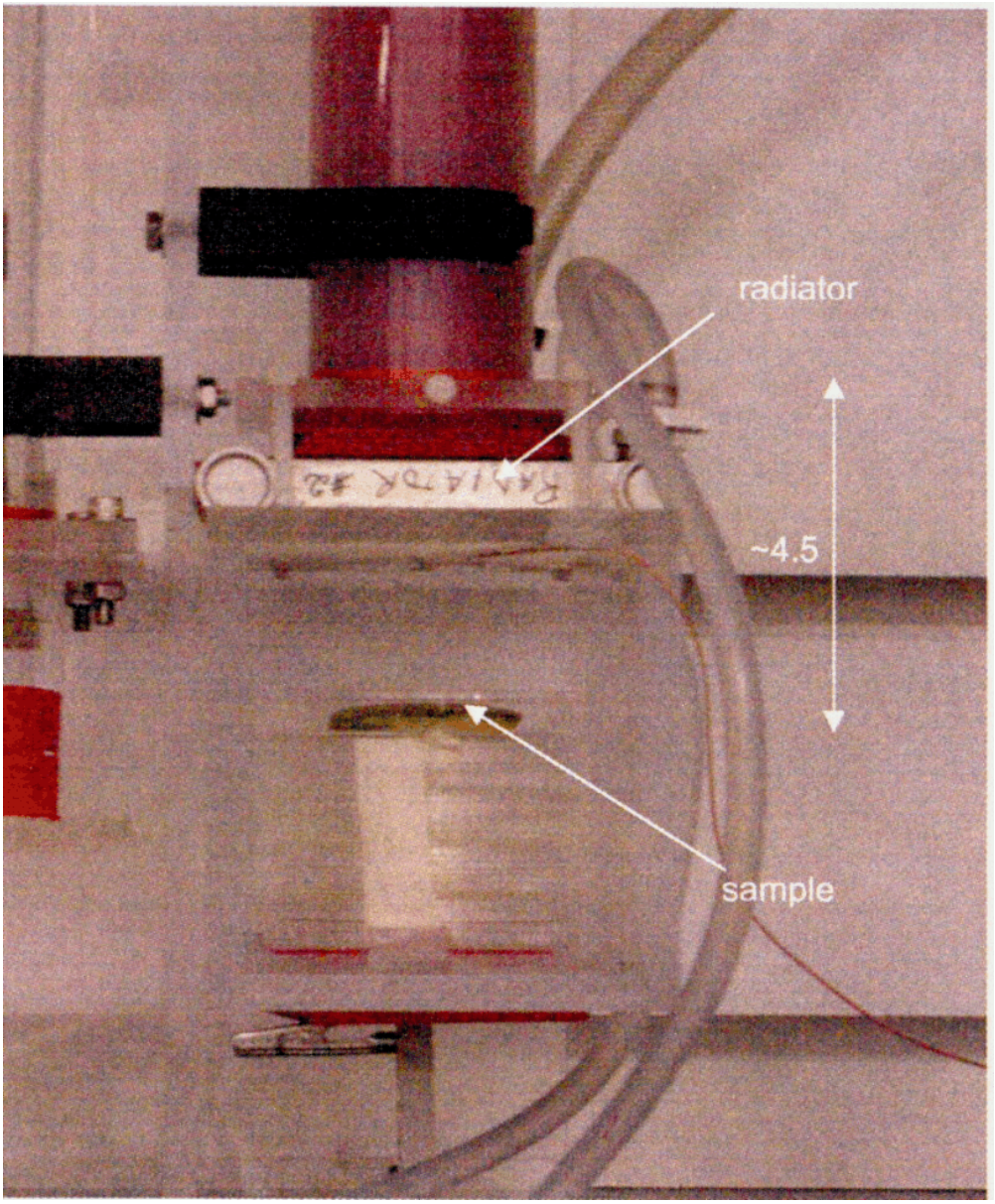

Figure 4. OAUGDP sample exposure chamber.

Table 1. Percent injury of surviving populations of target pathogens after treatment with the OAUGDP.

% injury[1] of target organism after treatment of inoculated:						
Exposure time (min)	*E. coli* on TSAN	*E. coli* on MEMB	*Salmonella* on Cantaloupe	*Salmonella* on TSAN	*Listeria* on Lettuce	*Listeria* on TSA
0	27.1	83.2	94.9	78.6	20.6	30.7
0.17	67.1	-[3]	-	92.7	-	91.0
0.5	26.0	95.7	-	94.8	-	97.7
1	74.3	36.8	92.3	52.1	65.1	CI[4]
2	-	78.1	-	-	-	-
3	-	-	81.7	-	93.4	-
5	-	-	83.7	CI	8.4	-

[1] % injury = $\frac{\text{CFU on non-selective media - CFU on selective media}}{\text{CFU on non-selective media}}$ x 100%

[2] Non-selective and selective media were: TSAN and MEMB for *E. coli* O157:H7; TSAN and XLT4 for *Salmonella*; TSA and MOX for *L. monocytogenes*.

[3] Not recovered

[4]Complete inactivation

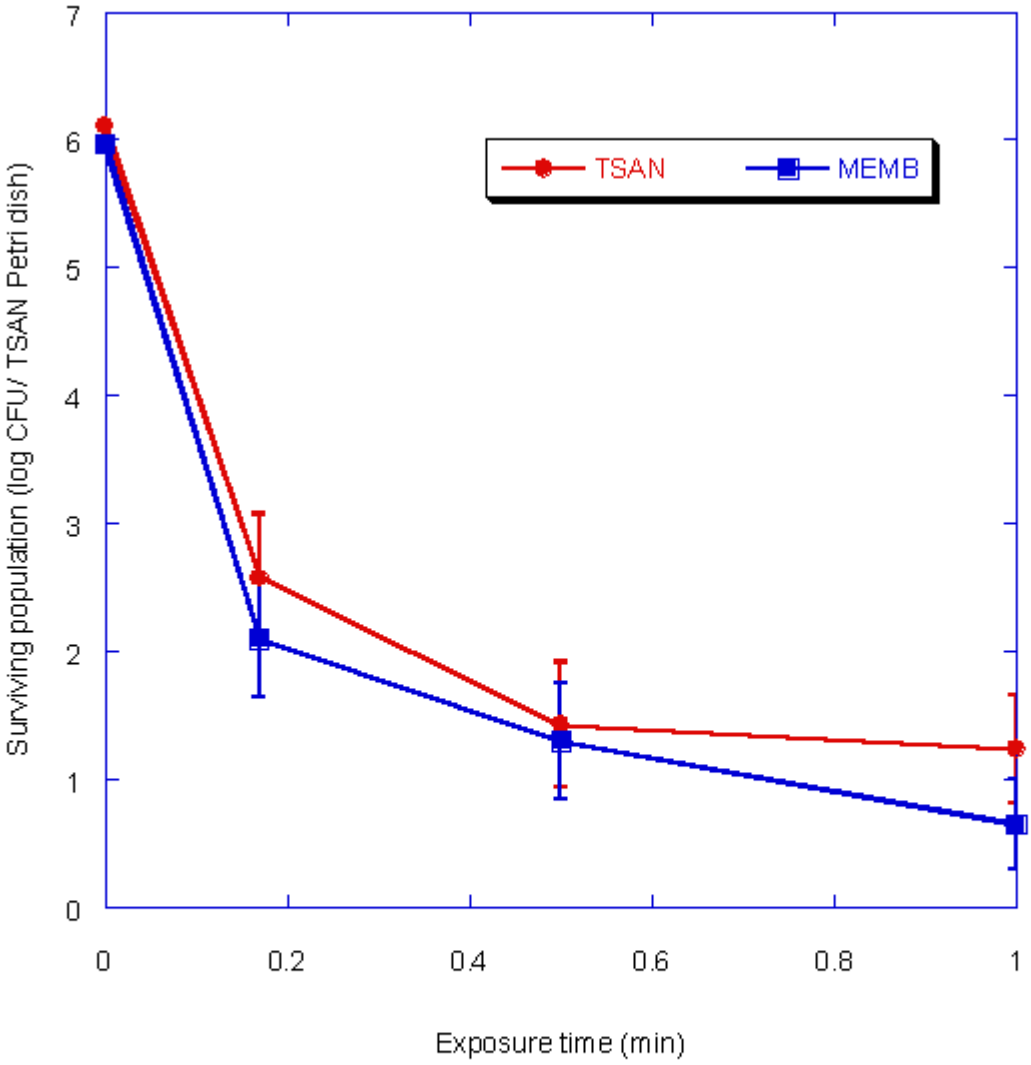

Figure 5. Survival of *E. coli* O157:H7 on TSAN exposed to the OAUGDP and recovered on TSAN and MEMB.

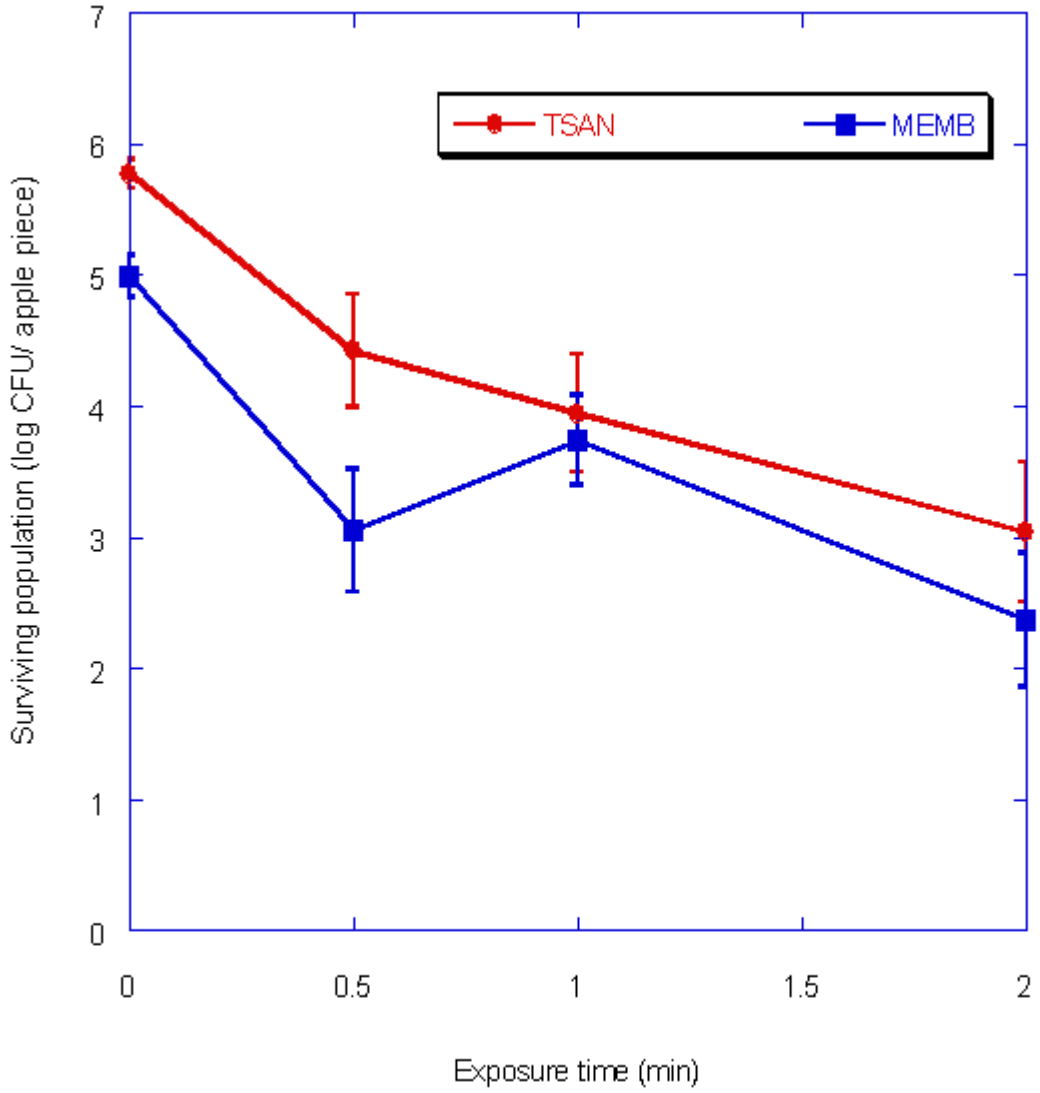

Figure 6. Survival of *E. coli* O157:H7 on red delicious apples exposed to the OAUGDP and recovered on TSAN and MEMB.

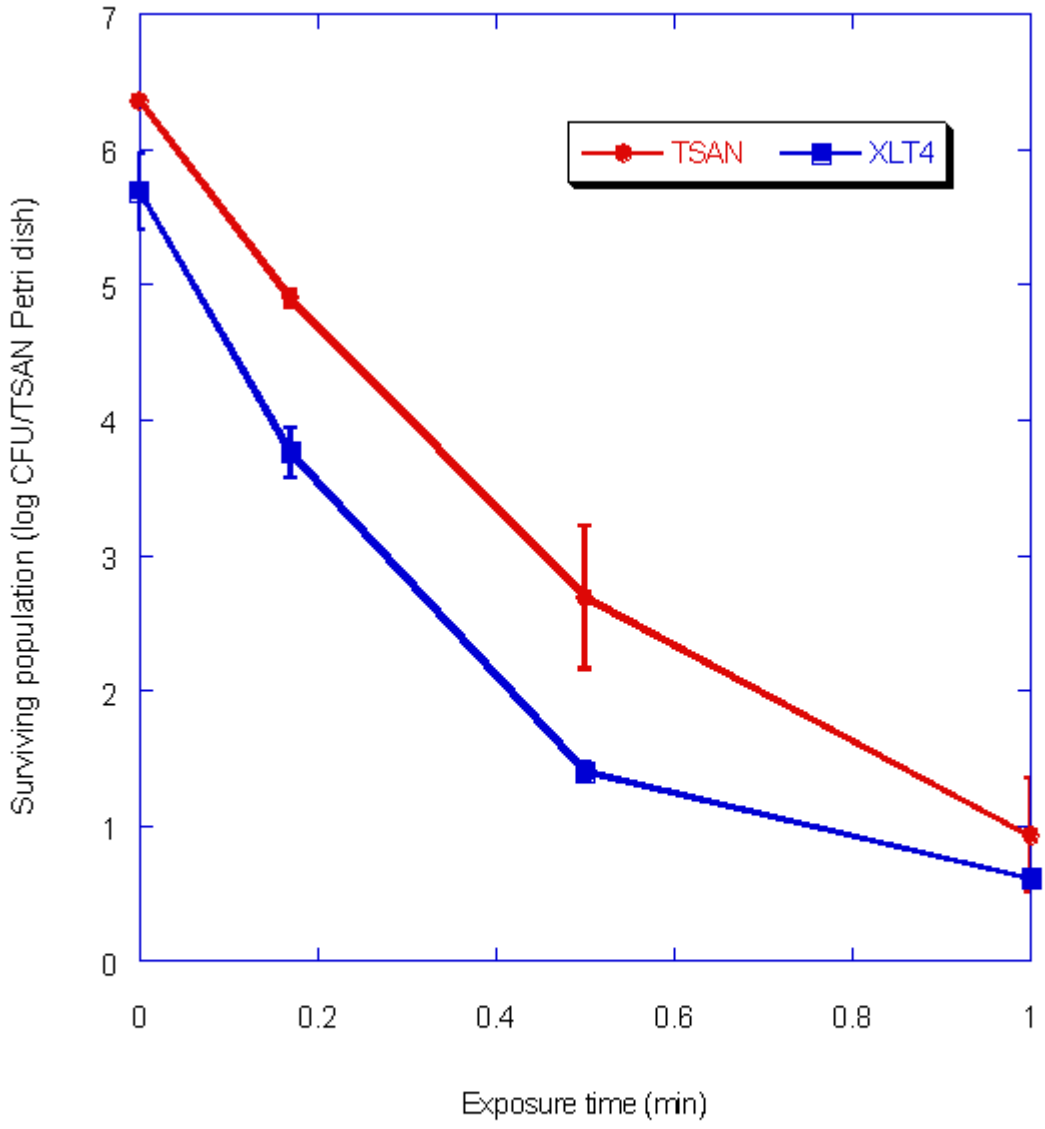

Figure 7. Survival of *Salmonella* on TSAN exposed to the OAUGDP and recovered on TSAN and XLT4.

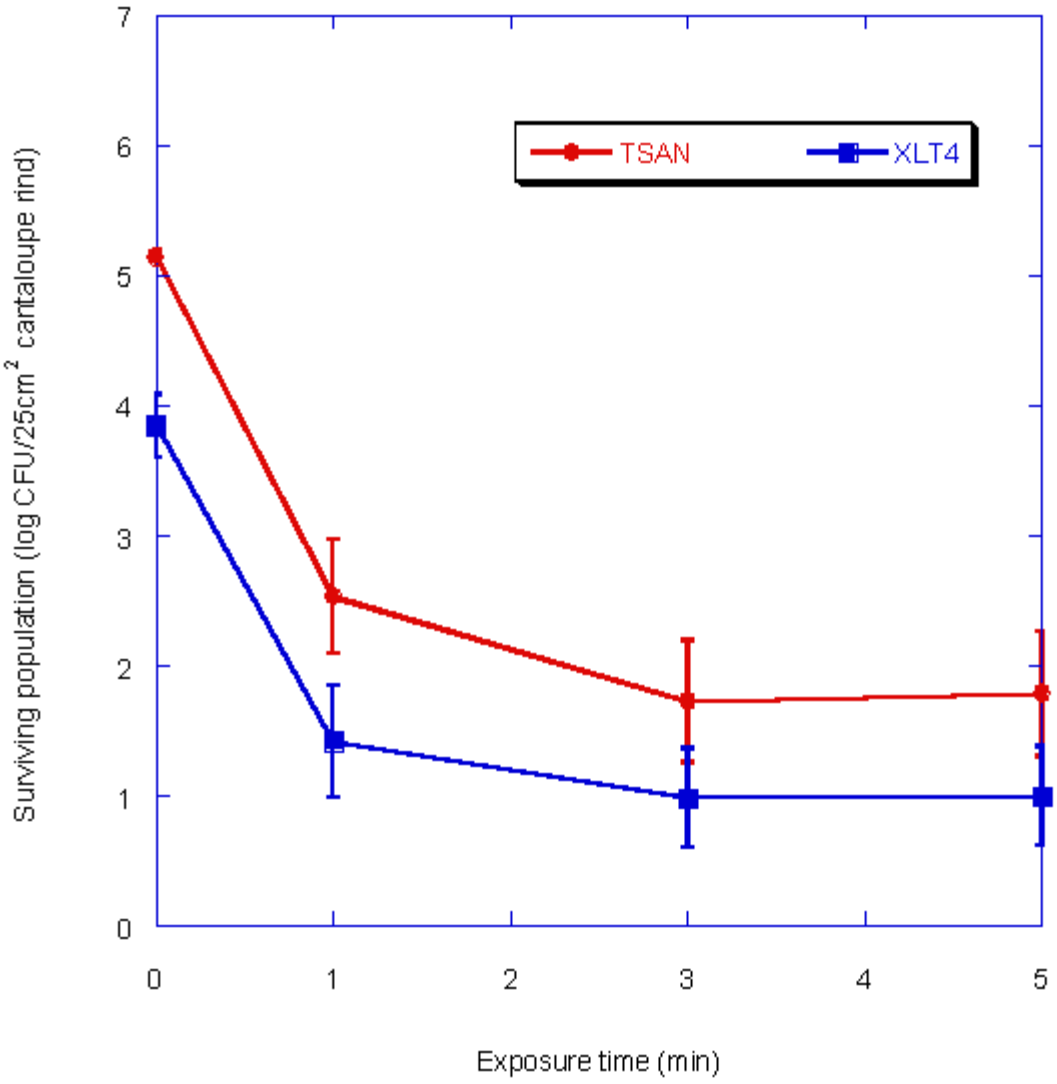

Figure 8. Survival of *Salmonella* on cantaloupe rinds exposed to the OAUGDP and recovered on TSAN and XLT4.

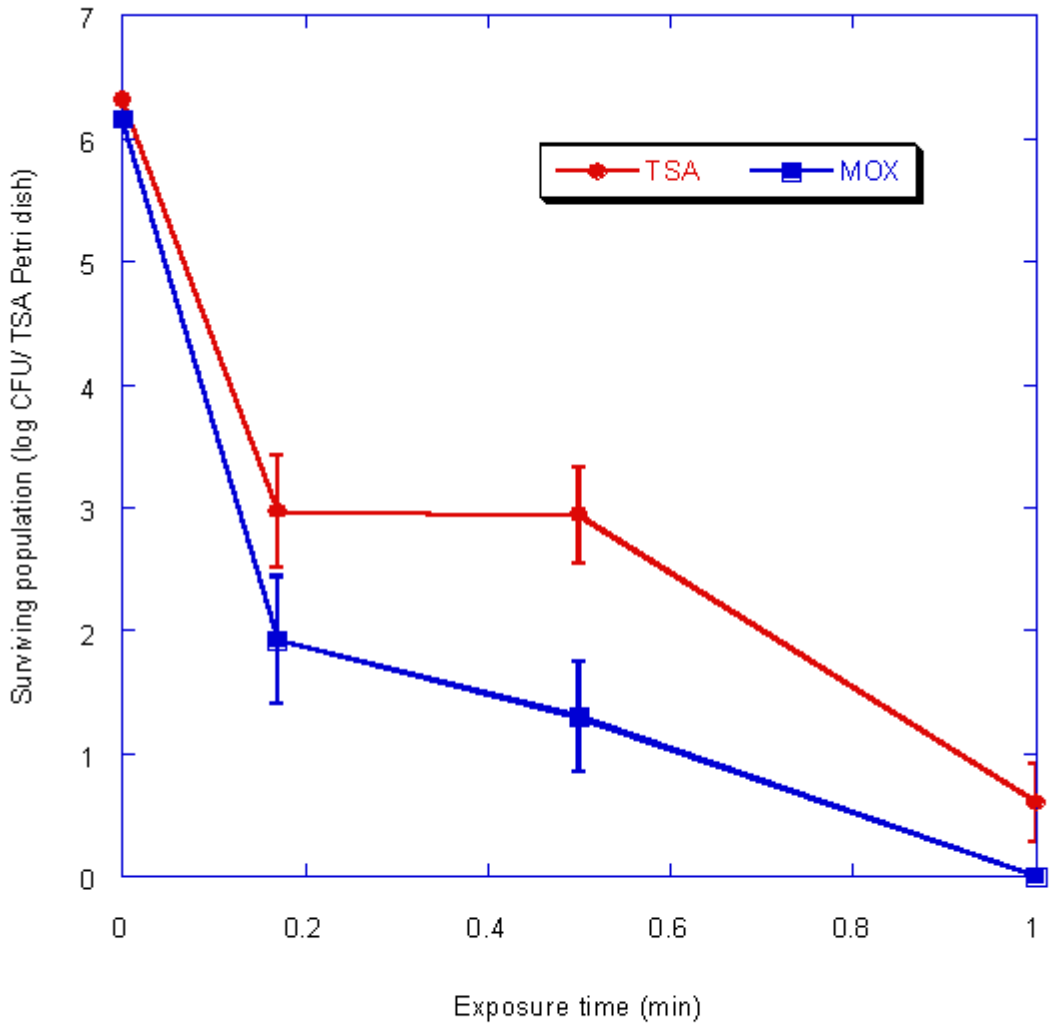

Figure 9. Survival of *L. monocytogenes* on TSA exposed to the OAUGDP and recovered on TSA and MOX. Values shown as 0 log CFU/TSA Petri dish represent no detection.

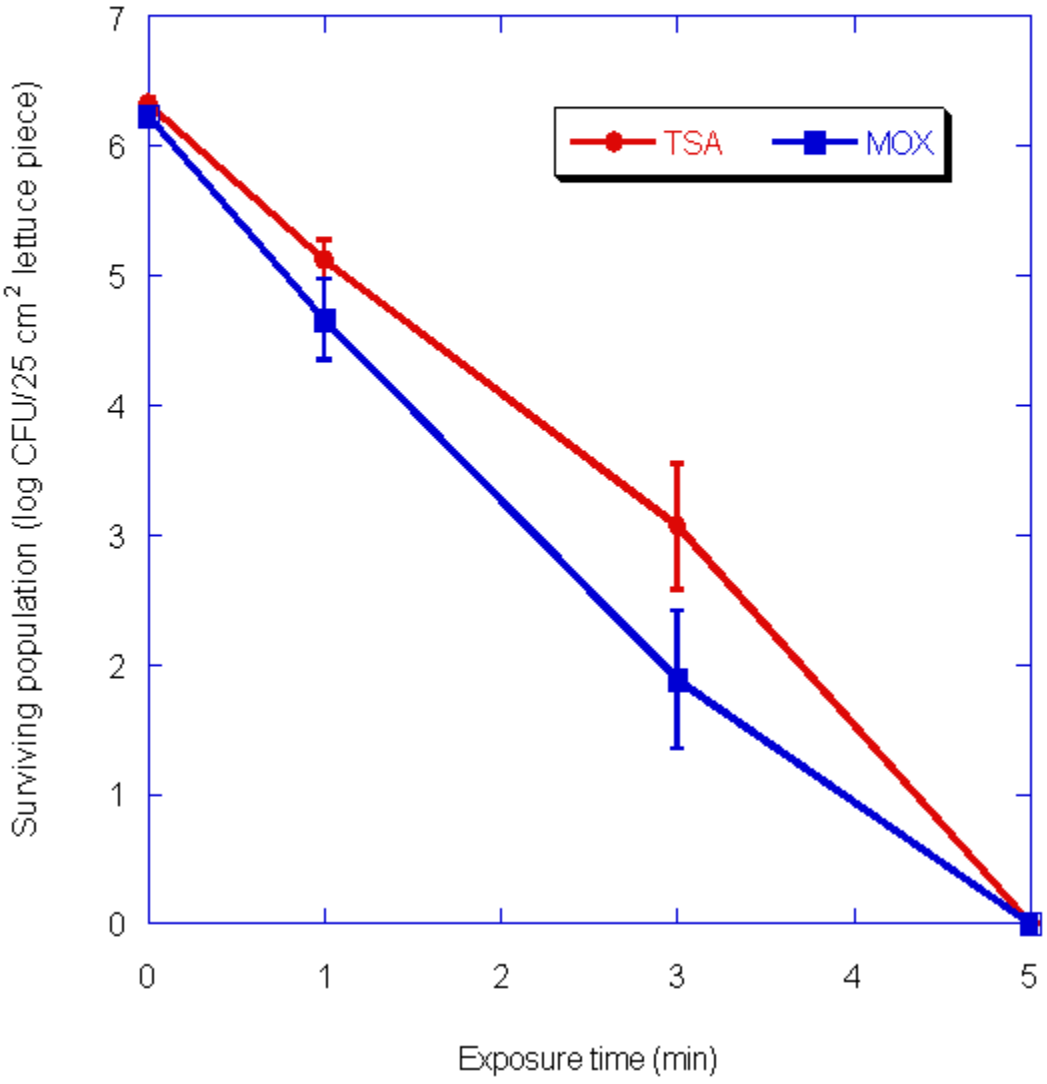

Figure 10. Survival of *L. monocytogenes* on iceberg lettuce leaves exposed to the OAUGDP and recovered on TSA and MOX. Values shown as 0 log CFU/25 cm^2 represent no detection.

SAS Programming: *E. coli* O157:H7 exposed on TSAN and recovered on TSAN

```
data one;
input time CFU;
datalines;

%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
macros\mmaov.sas';
%mmaov(one, CFU, class=time, fixed=time, adjust=tukey);
```

SAS Programming: *E. coli* O157:H7 exposed on TSAN and recovered on MEMB

```
data one;
input time CFU;
datalines;

%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
macros\mmaov.sas';
%mmaov(one, CFU, class=time, fixed=time, adjust=tukey);
```

SAS Programming: *E. coli* O157:H7 exposed on apple sections and recovered on TSAN

```
data one;
input time CFU rep;
datalines;

%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
macros\mmaov.sas';
%mmaov(one, CFU, class=rep time, fixed=time, random=rep, adjust=tukey);
```

SAS Programming: *E. coli* O157:H7 exposed on apple sections and recovered on MEMB

```
data one;
input time CFU rep;
datalines;
%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
```

```
macros\mmaov.sas';
%mmaov(one, CFU, class=rep time, fixed=time, random=rep, adjust=tukey);
```

SAS Programming: *Salmonella* exposed on TSAN and recovered on TSAN

```
data one;
input time CFU;
datalines;

%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
macros\mmaov.sas';
%mmaov(one, CFU, class=time, fixed=time, adjust=tukey);
```

SAS Programming: *Salmonella* exposed on TSAN and recovered on XLT4

```
data one;
input time CFU;
datalines;

%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
macros\mmaov.sas';
%mmaov(one, CFU, class=time, fixed=time, adjust=tukey);
```

SAS Programming: *Salmonella* exposed on cantaloupe rinds and recovered on TSAN

```
data one;
input time CFU rep;
datalines;

%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
macros\mmaov.sas';
%mmaov(one, CFU, class=rep time, fixed=time, random=rep, adjust=tukey);
```

SAS Programming: *Salmonella* exposed on cantaloupe rinds and recovered on XLT4

```
data one;
```

```
input time CFU rep;
datalines;

%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
macros\mmaov.sas';
%mmaov(one, CFU, class=rep time, fixed=time, random=rep, adjust=tukey);
```

SAS Programming: *L. monocytogenes* exposed on TSA and recovered on TSA

```
data one;
input time CFU;
datalines;

%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
macros\mmaov.sas';
%mmaov(one, CFU, class=time, fixed=time, adjust=tukey);
```

SAS Programming: *L. monocytogenes* exposed on TSA and recovered on MOX

```
data one;
input time CFU;
datalines;

%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
macros\mmaov.sas';
%mmaov(one, CFU, class=time, fixed=time, adjust=tukey);
```

SAS Programming: *L. monocytogenes* exposed on iceberg lettuce and recovered on TSA

```
data one;
input time CFU rep;
datalines;

%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
macros\mmaov.sas';
%mmaov(one, CFU, class=rep time, fixed=time, random=rep, adjust=tukey);
```

SAS Programming: *L. monocytogenes* exposed on iceberg lettuce and recovered on MOX

```
data one;
input time CFU rep;
datalines;


%include 'C:\Documents and Settings\Faith\My Documents\ANS571\sas
macros\mmaov.sas';
%mmaov(one, CFU, class=rep time, fixed=time, random=rep, adjust=tukey);
```

VITA

Faith Johnson was born in Nashville, Tennessee on August 4, 1978 to parents Dallas and Barbara Johnson. Faith grew up in Nolensville, Tennessee with her younger sibling Ryan Johnson and graduated from Page High School in 1997. She then moved onto the University of Tennessee at Knoxville where she received two B.S. degrees majoring in Animal Science and Food Science. In June 2003, she began the M.S. program in Food Science focusing in Food Microbiology and graduated from this program in August 2004. Faith has continued her education at the University of Tennessee pursuing her doctorate in Food Microbiology.